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(54) Title: REGULATION OF OSTEOCLAST FORMATION BY INHIBITION OF OSTEOBLASTIC STEM CELL FACTOR (57) Abstract The present invention provides an inhibitor of osteoblastic stem cell factor binding and/or activity, for example, an antibody or an antisense oligonucleotide. Also provided are pharmaceutical compositions comprising these inhibitors of osteoblastic stem cell factor binding and/or activity. Further provided is a method of regulating the activity of osteoclasts, comprising the step of: inhibiting the binding and/or activity of osteoblastic stem cell factor.		

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**REGULATION OF OSTEOCLAST FORMATION BY
INHIBITION OF OSTEOBLASTIC STEM CELL FACTOR**

10

BACKGROUND OF THE INVENTION

15 Cross-Reference to Related Application

This application claims benefit of provisional application
Serial No. 60/058,484, filed September 10, 1997, now abandoned.

Federal Funding Legend

20 This invention was created in part using funds from
National Institutes of Health grant number AG12951. The federal
government, therefore, has certain rights in this invention.

Field of the Invention

25 The present invention relates generally to the fields of
biochemical endocrinology and regulation of bone formation and
degradation. More specifically, the present invention relates to
regulation of osteoclast formation by inhibition of osteoblastic
stem cell factor.

Description of the Related Art

The human skeleton is continuously remodeled, normally turning over in ~2 years and allows use of skeletal mineral in calcium homeostasis. The strength and shape of the skeleton is preserved by segmental replacement: a bone section is degraded by osteoclasts, formed from monocyte-macrophage precursors,¹⁻² while osteoblasts, derived from stromal cells,³ synthesize new bone. These unrelated cells differentiate in a coupled manner, producing a new bone section in a few weeks.

Overall bone turnover responds to parathyroid hormone, but how differentiation of osteoblasts and osteoclasts is coordinated locally to maintain bone integrity is poorly understood. Osteoclast differentiation requires that precursors contact osteoblast-like cells,⁴ suggesting specialized recognition molecules. *In situ* unlabeled antibody and Western blot analysis revealed that osteoblasts express a surface-bound form of stem cell factor (SCF; *c-kit* ligand) during bone synthesis only. Stem cell factor production in isolated osteoblasts responds to parathyroid hormone. Differentiation of osteoclasts from monocytes is supported by osteoblast-derived stem cell factor-producing cells *in vitro*, a process interrupted by antibody or antisense oligonucleotide targeting stem cell factor, indicating that it is a key element controlling this process.

The SCF/*kit* signaling pathway is very complex. Briefly summarized, stem cell factor binding induces receptor dimerization, which is associated with phosphorylation. Activity is transduced through intracellular kinases of the *src* family, the oncogene *c-Cbl* and pI-3 kinase. *Src* and *Cbl* are required for

osteoclast differentiation; *src*, *Cbl* and PI-3-kinase interact with other osteoclast signaling molecules.

When ionized calcium is suppressed, such as with retention of phosphate in kidney failure, parathyroid hormone is secreted in large quantities and bone turnover increases as much as ten-fold, coupling of bone formation and degradation is maintained. Occurrence of stem cell factor in bone of hyperparathyroid subjects was examined because abnormal mast cell differentiation occurs around bone trabeculae of these patients,⁵ and this protein causes mast cell differentiation in vitro.⁶ Stem cell factor is expressed in a variety of forms in several tissues,⁷ with a soluble form produced by a six-exon transcript and a longer membrane-associated form produced by an eight-exon transcript.⁸ Additional variation occurs with proteolytic cleavage and glycosylation.

The prior art is deficient in the lack of effective means of inhibiting osteoclast formation and activity and thereby regulating bone formation and/or degradation. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention discloses, *inter alia*, that an antibody to a conserved region of the C-terminus of the *c-kit* ligand completely blocks the formation of human osteoclasts. This region is present in membrane-bound and some secreted forms of the protein, but is absent from other reported forms of the protein and may not required for some functions of *c-kit* ligand.

Furthermore, in a controlled in vitro system with only osteoblast-like stromal cells and monocytes, osteoclast formation is blocked by antisense nucleotides or antibodies to stem cell factor and thus is a unique limiting factor coupling bone cell differentiation.

5 Osteoclasts mediate bone degradation that is responsible for osteoporosis, bone lesions in metastatic cancer, and other disease states. Thus, an application of the present invention is to control osteoclast formation, and therefore bone loss, in disease states of bone loss.

10 Using a *c-kit* ligand to reduce bone degradation has the particular advantage of inhibiting a system that affects, in (receptor) deficient *mi/mi* mice, only mast cells, and therefore has limited or no toxicity. Further, use of C-terminal blocking to inhibit osteoclast activity uses a portion of the molecule with no
15 other known function.

 Thus, use of antibodies or antisense nucleotides specific for the *c-kit* ligand, can reduce or prevent bone loss in aging (osteoporosis) or cancer progression (metastatic bone disease). These compounds target both the formation (tartrate-resistant acid phosphatase-positive cells) and the activity (bone
20 resorption) of osteoclasts.

 In one embodiment of the present invention, there is provided an inhibitor of osteoblastic SCF binding and/or activity.

 In another embodiment of the present invention, there
25 is provided a pharmaceutical composition, comprising an inhibitor of osteoblastic stem cell factor binding and/or activity and a pharmaceutically acceptable carrier.

 In yet another embodiment of the present invention, there is provided a method of regulating the activity of

osteoclasts, comprising the step of: inhibiting the binding and/or activity of osteoblastic stem cell factor.

In still yet another embodiment of the present invention, there is provided a method of treating a pathophysiological state in an animal in need of such treatment, wherein the pathophysiological state involves bone loss, comprising the steps of: administering a pharmaceutical composition disclosed herein to the animal.

In another embodiment of the present invention, there is provided a method of diagnosing bone disorders, comprising the step of measuring the activity of osteoblastic stem cell factor.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the SCF production in human bone cells. Figures 1A-1D shows stem cell factor in bone biopsy sections. Seven μm sections of formalin fixed, methacrylate embedded tissue were de-plasticized and stem cell factor was identified by the unlabeled antibody technique, with hematoxylin counter-stain to show cellular detail. Stem cell factor in single sections from hyperparathyroid (Figures 1A,1D) and normal (Figures 1C,1D) patients demonstrate reaction (brown color) of the antibody with cuboidal active osteoblasts (ob) (Figures 1A and 1C) in a membrane pattern (arrows). Quiescent, attenuated osteoblasts (Figures 1B and 1D) were nonreactive. Irrelevant hybridoma supernatant was nonreactive (not illustrated). Scale bars, 5 μm . Figure 1E shows a Western blot analysis of bone trabecular and marrow cells. Vertebral trabecular bone from patient with rapid bone loss (autopsy tissue from a 52 year old female on cortisol therapy) was separated from marrow by vortexing and 100 μg aliquots of protein separated on SDS-PAGE for Western blot analysis as described.⁸ Non-reactive hybridoma supernatant and marrow cell protein were not labeled by the antibody, but bone cell proteins at M_r ~45 and ~33 kD were labeled. Results are representative of several reactions.

Figure 2 shows the stem cell factor production by osteoblast-like cell lines and osteoblasts. Figure 2A shows a Western blot analysis of SaOS2 human osteosarcoma cells with indicated hormones during 5 day pre-treatment; stem cell factor production was very low unless parathyroid was present; 1,25-dihydroxyvitamin D had no effect. Unlike normal bone cells (see Figure 1E), only one form, M_r ~45 kD, was observed. Figure 2B

shows an ELISA assay of UMR-106 cells. This rat osteoblast-like line, at passage 10, was treated for 120 hours with 10^{-9} M parathyroid hormone (PTH), 10^{-8} M 1,25-dihydroxyvitamin D (D), or both. For the ELISA assay, microtiter wells were coated with 5 μ g of protein; bound human anti-stem cell factor was determined using alkaline phosphatase-coupled anti-rabbit serum (Bio-Rad, Richmond, CA) and p-nitrophenol- PO_4 substrate, with absorbance at 550 nm. Quadruplicate results \pm standard deviation are shown in this and subsequent quantitative assays. Time course showed minimal parathyroid hormone effect at 1-3 days pre-incubation and Western analysis was similar to Figure 1E (not illustrated). Figure 2C shows a Northern blot analysis of stem cell factor in isolated human osteoblasts. Cells were pre-treated 120 hours in indicated conditions. Two mRNAs are detected, just as two sizes of the protein are present in non-transformed human osteoclasts (Figure 1C). MG63 RNA is a positive control (right lane); osteoblasts produce barely detectable levels of mRNA (left lane) except when 10^{-9} M parathyroid hormone is added (second lane), a process unaffected by glucocorticoids (third lane).

Figure 3 shows the effect of blocking stem cell factor on osteoclast production. Figure 3A-D shows tartrate-resistant acid phosphatase (TRAP)-positive cells derived from macrophages cultured with MG63 cells and effect of antibody to stem cell factor. Figure 3A shows the human macrophages in 2 cm^2 tissue culture wells incubated 14 days with $0.5 \text{ }\mu\text{g/ml}$ recombinant CSF-1 (Genzyme, Cambridge, MA) added at 3 day increments (required for cell viability in absence of stromal cells). TRAP, a characteristic product of osteoclast differentiation, is not present,

although many giant cells have formed. **Figure 3B** shows the macrophages as in **Figure 3A** co-cultured 14 days with MG63 cells (passage 43, 10^4 per cm^2 at day 0 and near-confluent at day 14). In this control culture, pre-immune rabbit serum at 1:100 dilution was added to control for the rabbit serum addition in **Figure 3D** below. Note that the giant cells formed are strongly TRAP positive (red color). **Figures 3C-3D** shows the anti-stem cell factor antibody at 1:500 and 1:100 dilution respectively, with dose-dependent decrement in staining. Bars: 20 μm . **Figure 3E** shows the effect of stem cell factor antibody on TRAP activity in 14 days co-cultures of human macrophages with MG63 (left) or SaOS2 (right) cells. Pre-immune rabbit serum (1:25, PICS) control and a wide range of serum concentrations are tested using MG63 and SaOS2 cells to support osteoclast differentiation. Antibody (Ab) concentration-dependent reduction of TRAP occurs in both cases. Macrophage (10 μg , MF) and chicken osteoclast²¹ lysates (2 μg , OC) are positive and negative controls. **Figure 3F** shows the degradation of bone by osteoclasts formed in 14 day co-cultures of human macrophages and MG63 or SaOS2 cells, and effect of antibody to stem cell factor, assayed using 20 μg of ^3H labeled substrate, measuring label released into the supernatant.²¹ **Figures 3G and 3H** shows the effect of stem cell factor antisense phosphorothioate oligonucleotide and sense control on formation of TRAP and bone degradation, using MG63 cells with human macrophages and 1.5 μM sense or antisense oligonucleotides as indicated.

Figure 4 demonstrates antibody staining in living MG63 cells. A patchy surface staining pattern is seen (arrows, left

panel). The reaction can be successfully inhibited by pre-incubation of the antibody with excess antigen (middle panel). Incubation with pre-immune serum results in only faint background fluorescence.

5 **Figure 5** depicts a Western blot analysis of stem cell factor production with sense/antisense treatment. In this figure, the form of stem cell factor at 45 kDa is seen clearly. Consistent inhibition was seen at time periods greater than five days following treatment with 1.5 μ M antisense oligonucleotide.

10 **Figure 6** illustrates MG63 cells stably transfected with pCDNA3 containing antisense to the translation start site of human *c-fms* ligand driven by the CMV promoter. **Figure 6A** shows the transfected MG63 cells growing in the neomycin analog G418. Transfected cells at this point had colonies (left panel),
15 while the controls with empty vector had no living cells (right panel). **Figure 6B** is a Western analysis showing expression of the ~36 and 50 kDa forms of stem cell factor in MG63 with vector only (vector controls V3, V6) and wild type MG63 (middle lane), but not in transfectants expressing stem cell factor antisense
20 (Antisense 13, 17).

Figure 7 shows a comparison of the C-terminal sequences (with region for antibody production boxed) of several proteins.

25 **Figure 8** shows analysis of the human stem cell factor protein, with the region used for antibody development highlighted. The region selected is conserved absolutely in mammals (E/Q and E/D substitutions in first three amino acids in lower species).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an inhibitor of osteoblastic SCF binding and/or activity. In one aspect, the inhibitor is an antibody. Preferably, the inhibitor is directed against the membrane associated form of osteoblastic SCF and the antibody is directed against the C-terminal end of the SCF protein. In a preferred embodiment, the antibody is directed against the C-terminal end comprises the decapeptide, EEDNEISMLQ (SEQ ID No.:1). Both human and non-human forms of the antibody can be employed for the various uses described herein. In another aspect, the present invention relates to an antisense oligonucleotide directed against expression of the stem-cell factor. Preferably, the antisense oligonucleotide is directed against the stem-cell factor transcription start site.

In another aspect, the present invention relates to a method of regulating the activity of osteoclasts, comprising the step of: inhibiting the binding and/or activity of osteoblastic stem cell factor. Both the antibodies and the antisense oligonucleotides described above are useful in this method.

It is specifically contemplated that pharmaceutical compositions may be prepared using the antibodies and the antisense oligonucleotides of the present invention. In such a case, the pharmaceutical composition comprises the antibodies and the antisense oligonucleotides of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages and routes of

administration of the antibodies and the antisense oligonucleotides of the present invention.

In therapeutic applications, the oligonucleotides are utilized in a manner appropriate for treatment of a variety of conditions by inhibiting expression of the target genetic regions. For such therapy, the oligonucleotides alone or in combination can be formulated for a variety of modes of administration, including systemic, topical or localized administration. Techniques and formulations generally can be found in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa. The oligonucleotide active ingredient is generally combined with a pharmaceutically acceptable carrier such as a diluent or excipient which can include fillers, extenders, binders, wetting agents, disintergrants, surface active agents or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions, and solutions, granules, capsules and suppositories, as well as liquid preparations for injections, including liposome preparations.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal and subcutaneous. For injection, the oligonucleotides of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers. In addition, the oligonucleotides can be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. Dosages that can be used for systemic administration preferably range from about 0.01 mg/kg to 50 mg/kg administered once or twice per day. However, different dosing schedules can be utilized

depending on: (1) the potency of an individual oligonucleotide at inhibiting the activity of its target DNA; (2) the severity or extent of the pathological disease state; or (3) the pharmacokinetic behavior of a given oligonucleotide.

5 In another aspect, the present invention relates to a method of treating a pathophysiological state in an animal in need of such treatment. A representative pathophysiological state would be one that involves bone loss. This method comprises the steps of: administering a pharmaceutical composition disclosed
10 herein to said animal. Preferably, the pathophysiological state is selected from the group consisting of osteoporosis, bone cancer and malignant hypercalcemia.

 In another aspect, the present invention relates to a method of diagnosing bone disorders state in an individual,
15 comprising the step of measuring the activity of osteoblastic stem cell factor in a sample taken from said individual. Representative bone disorders which can be diagnosed using this technique include osteoporosis, hyperparathyroidism, metastatic cancer and hypercalcemia.

20 In one embodiment of the present invention, a diagnostic use of stem cell factor assays can be employed for the diagnosis of bone disorders. This assay is an inexpensive serum-based test.

 A person having ordinary skill in this art would also
25 recognize that the immuno-therapeutic potential of an antibody to block stem cell factor activity. Such immunotherapy could be useful for malignant hypercalcemia where short term control is important and may be much less toxic than what is used now, e.g., (1) gallium nitrate, (2) a large number of bisphosphonate

derivatives, (3) antibiotics inhibitors herbimycin and plicamycin (plicamycin is also known as mithramycin, and has been abandoned due to severe toxicity), (4) bone-binding antibiotics, principally tetracyclines, (5) proteinase inhibitors, (6) estrogen analogues or inhibitors (raloxifene, tamoxifen), and (6) tyrosine kinase inhibitors (genistein, herbimycin).

Another aspect of the present invention involves attacking normal stem cell factor ligand binding using peptide inhibitors. This could include recombinant stem cell factor (need not be human) with defects added to make a form that will bind to, but not activate, receptors. This peptide inhibitor would preferably be in the 300-500 kDa range. Alternatively, one may attack the system using low-molecular weight inhibitors that are either much smaller peptides or are non-peptide molecules. This approach would involve crystallizing the long form of SCF for detailed molecular analysis.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Development of antibody to a conserved sequence of the c-kit ligand

Monoclonal antibody 7H6 to human stem cell factor (Amgen, Thousand Oaks, California) was used for Figure 1. Western blot analysis⁸ in Figure 2 used rabbit polyclonal anti-human stem cell factor (Medical and Biological Laboratories, Nagoya, Japan).

The antibody to a hydrophilic, antigenic portion in the C-terminal stem cell factor was made in isolation from truncated (soluble) forms of the molecule.⁸ The decapeptide, EEDNEISMLQ (SEQ ID No.:1), nine residues from the C-terminal of human stem cell factor, was synthesized as a multi-antigen peptide on a branched lysine core (Research Genetics, Huntsville, AL). Rabbit antibodies were generated using Freund's adjuvant; selected antiserum reacted in ELISA at 1:10,000 dilution, and recognized the M_r ~45 kD form of SCF on Western blots. Antigen-inhibitable reaction with living MG63 cells showed that this epitope is exposed on these cells.

Amino-acid sequences of stem cell factors from dog (GenBank accession S53329), chicken (SC), human (M59964), Japanese quail (U43078, U43079) and mouse (M57647) were aligned by the clustal method. A hydrophilic, antigenic decapeptide, EEDNEISMLQ (SEQ ID No.:1), nine residues from the C-terminal end of the stem cell factors, was selected for antibody production. This region is identical in the five species except for E/Q or D/E substitutions in the avian species in its leading three acidic residues. It was chosen based on its position in the molecule, is present in both soluble- and membrane-forms of stem cell factor, and is the best conserved region with very high Kyte-Doolittle hydrophilicity score, Jameson-Wolf antigenic index, and Emini surface probability. Further, this region is centered in a beta amphipathic region with turns, a combination of features designed to select a surface region involved in protein-protein interactions. Analysis used Lasergene bio-computing software (DNASTAR, Madison, WI).

The selected region was synthesized as a multi-antigen peptide on a branched lysine core (Research Genetics, Huntsville, AL) and used with Freund's adjuvant with primary and two booster doses to generate antibodies in two rabbits, with one
5 selected for further use on the basis of enzyme-linked immune assay titers and Western analysis results.

EXAMPLE 2

Oligonucleotide synthesis

10 A cDNA probe for stem cell factor mRNA was made by reverse transcription and polymerase chain reaction using RNA from MG63 cells and primers GCCTTTCCTTATGAAGAAGAC (SEQ ID No.:2) and TGCTGTCATTCCTAAGGGA (SEQ ID No.:3) to produce a
15 633 bp segment from -10 to 633 relative to transcription start (GenBank M59964), product identity confirmed by restriction digestion. Sense and antisense phosphorothioate oligonucleotides were made using the first primer sequence and its complement (CGGAAAGGAATACTTCTTCTG) (SEQ ID NO.:4).

EXAMPLE 3

Northern blot analysis

20 For Northern analysis, RNAs were isolated by phenol-GITC extraction; 5 µg aliquots were separated on agarose and transferred to nitrocellulose for hybridization. The 633 bp
25 segment of human stem cell factor cDNA, labeled with ³²P by random priming, was denatured and hybridized to the blot at 42°C overnight.¹⁸ Membranes were washed in 300 mM NaCl, 50 mM Na citrate, pH 7.0 15 minutes at room temperature (25° C), and

twice in 30 mM NaCl, 5 mM Na citrate, pH 7.0, 15 minutes, 65°C, and autoradiographed.

EXAMPLE 4

5 Cell culture and enzyme assays

Human macrophages were isolated by apheresis of volunteers, selecting surface-attached (1 hour), >99% non-specific esterase positive, cells.¹⁹ Each assay used a single macrophage preparation. Human osteoblasts were produced from medullary bone from surgical waste,²⁰ grown to confluence in Eagle's minimal essential with 10% heat-inactivated fetal bovine serum and 1 μ M cortisol, and used when mineralizing nodules appeared (~3 weeks). Mean \pm standard deviation, n=4. TRAP was measured as described.²¹ Media were replaced at 3 day intervals except for antisense assays where they were replaced every 2 days. For quantitative assays, TRAP was determined as absorbance at 540 nm.

EXAMPLE 4

20 Stem cell factor expression on the surfaces of synthetic osteoblasts

Stem cell factor was markedly expressed on the non-matrix surfaces of synthetic osteoblasts, but stem cell factor expression was not detected on quiescent osteoblasts (Figures 1A, 1B). Because the association with osteoblast activation suggested an important function for this cytokine, normal bone was also examined (Figures 1C, 1D). The pattern was the same, although active osteoblasts were, as expected, a much smaller proportion of the bone lining cells.

Western blot analysis showed that stem cell factor associated with bone lining cells was predominately the large form, although smaller forms were also present (Figure 1E). Production of stem cell factor production by bone-forming osteoblasts suggested that this factor is important in bone turnover. When turnover is amplified in hyperparathyroidism, formation of mast cells may occur as a side-effect.

The present invention shows that osteoblast surface stem cell factor plays a role in terminal differentiation of osteoclast precursors and this is the principal process linked to bone formation. It was known that pre-osteoclasts bear *c-kit*,⁹ and osteoblast-like cells that support osteoclast differentiation *in vitro* produce stem cell factor.¹⁰ Further, *mi/mi* mice, which have no mast cells and are osteopetrotic, have a defect in the *W* locus that produces *c-kit*.¹¹ Osteoblastic regulation of stem cell factor under controlled conditions was shown *in vitro*, using defined populations of human blood monocytes² and osteoblast-like stromal cells¹².

20

EXAMPLE 5

Production of SCF by osteoblast-like cells

To define the properties of the system, the production of stem cell factor by human and rat osteoblast-like cells *in vitro* was first determined. As seen in SaOS-2 human osteosarcoma cells, the production of stem cell factor was in some cases responsive to parathyroid hormone, but did not respond to 1,25 dihydroxyvitamin D, a steroid required for normal bone differentiation (Figure 2A, 2B). Osteoclasts and osteoclast

precursors did not produce detectable stem cell factor (not illustrated). Production of stem cell factor by nontransformed human osteoblasts *in vitro*, and parathyroid hormone activation, was also confirmed (Figure 2C) to provide a more controlled demonstration of the findings *in situ* (Figure 1).

ELISAs (Figure 2B) of the rat osteoblast-like cell line, UMR-106, at passage 10, indicate that vitamin D has a negative effect on stem cell factor production. PTH, conversely, is seen to drive stem cell factor to high levels. For the ELISA, micro-titer wells were coated with 5 µg of target cell protein, and bound anti-human stem cell factor was determined, using alkaline phosphatase-coupled anti-rabbit serum (BioRad, Richmond, CA) and p-nitrophenol substrate, absorbance at 550 nm. Blank solutions of matched volumes were used as controls. These assays were run in quadruplicate in 96-well plates. These results indicate that stem cell factor production in osteoblast-like cell lines, including the non-human line UMR-106, is similar to normal osteoblasts, but with varying hormonal responses.

20

EXAMPLE 6

Immunofluorescent detection of SCF in live cells

For the staining observed on living MG63 cells (Figure 4), cells were prepared as for Northern or Western analysis. They were then passed to 10 cm² wells in 6-well plates and grown on 25 mm coverslips. The protein localization via immunofluorescence used permeabilized fixed cells. The stem cell factor antibody was labeled with fluorescein. Antibody, antibody plus excess antigen, or preimmune serum at 1:100 were incubated for 30 minutes with living cells. The cells were then lightly fixed and bound antibody

visualized with fluorescein-conjugated goat-anti-rabbit serum as analyzed by epifluorescence microscopy. Fluorescent detection of stem cell factor in MG63 cells is shown in Figure 6E.

5

EXAMPLE 7

Antibody blockade of SCF

Osteoblast-like cells induce production of tartrate-resistant acid phosphatase (TRAP), an osteoclast marker, in fusing human monocytes in co-culture one week, which was blocked in a dose-dependent manner by addition of antibody to stem cell factor (Figures 3A-3D). The antibody used was directed at a conserved region of the protein unique to the membrane associated form. Reaction of antibody with the surface of living MG-63 cells, which was blocked by pre-incubation of antibody with antigen, showed that this epitope is exposed in the cells studied (Figure 4).

For these studies, one MG63 cell preparation and one macrophage preparation were used. Cultures were in 2 cm² cells on one 24 well plate, with 50 mg/cm devitalized bone. For the culture of macrophages without MG63 coculture, 0.5 µg/ml recombinant CSF-1 (Genzyme, Cambridge, MA) added at 3 day increments (required for cell viability in absence of stromal cells). The MG63 cells that were co-cultured with the macrophages were at passage number 43, with a cell density of 10⁴ per cm² at day 0 and near-confluent at day 14. The antibody employed for stem cell factor detection was the decapeptide, EEDNEISMLQ (SEQ ID NO: 1), as described *supra*.

The effects of a blocking antibody were further characterized by measuring TRAP and degradation of labeled bone

substrate by osteoclasts formed. Consistent antibody dose dependent complete inhibition of the osteoclast-specific enzyme (TRAP) or bone degradation were noted, with similar results in co-cultures using parathyroid hormone-responsive (SaOS2) or
5 unresponsive (MG63) osteoblast-like support cells (Figures 3E-3F). Pre-immune serum had no effect, and there was no effect on supporting cell density or appearance. Although bone regeneration is complex and involves many control mechanisms, the blocking data as shown in Figure 3 suggests that controlled
10 expression of cell-surface stem cell factor is central to matching bone synthesis and degradation.

EXAMPLE 7

Antisense oligonucleotide inhibition of SCF expression

15 Antisense oligonucleotides were used as an independent method to reduce stem cell factor expression. Antisense or sense (control) phosphorothioate oligonucleotides centered on the stem-cell factor transcription start site were added to co-cultures of osteoclast precursors and supporting cells.
20 Media were replaced, including oligonucleotides, every two days. The antisense oligonucleotide reduced osteoclast activity or TRAP production 20-40% at 0.5 μ M and nearly totally at 1.5 μ M (Figures 3G-3H). These results indicate that stem cell factor produced by osteoblasts is required for terminal differentiation of the
25 osteoclast. As an additional control, oligonucleotide binding to the stem cell factor translation start site was confirmed by use of the antisense sequence for PCR amplification of SCF cDNA .

EXAMPLE 8

Elimination of target expression in MG63 cells by stable transfection

5 The oligonucleotide technique suffers from a number of limitations, including incomplete inhibition and toxicity. A more definitive approach is to transfect MG63 cells with plasmids to produce cells with varying expression of membrane stem cell factor. Plasmids were constructed that express stem cell factor
10 sense and antisense RNA, MG63 cells were stably transfected with these and Western analysis was performed to demonstrate variable stem cell factor levels in the resultant cell lines (Figure 6B). Assays of osteoclasts formed in co-cultures with stem cell factor-positive and stem cell factor-negative cells yielded results
15 similar to those seen in Figure 3G and 3H.

 MG63 cells were stably transfected with pCDNA3 containing antisense to the translation start site of human *c-fms* ligand driven by the CMV promoter. A 633 base pair (bp) partial *c-fms* ligand cDNA was placed in the eukaryotic expression vector
20 pCDNA3 (Invitrogen, San Diego, CA) in antisense orientation at Eco RI and Xba I sites; this construct was confirmed by sequencing. Vector elements included the enhancer-promoter for the immediate early gene of CMV as well as polyadenylation/transcription termination sites to improve RNA
25 stability, neomycin resistance for selection in eukaryotes and SV40 *ori* for episomal replication. Empty vector and vector with antisense stem cell factor were transfected by the cationic lipid method (Tfx 20, Promega, Madison, WI) with 0.5 µg vector and 3:1 charge excess of cationic lipid. Incubation with 60% confluent

MG63 cells at 37° C for one hour followed, then selection in 100 µg/ml G418 (a neomycin) was carried out for ten days.

The following references were cited herein:

1. Scheven, et al., *Nature*. **321**, 79-81, 1986.
- 5 2. Fujikawa et al., *Endocrinol.* **137**, 4058-60 (1996).
3. Rickard, et al., *J. Bone Mineral Res.* **11**, 312-24, (1996).
4. Jimi, et al., *Endocrinol.* **137**, 2187-90 (1996).
5. Rockoff, et al., *Calcified Tissue Res.* **5**, 49-55 (1970).
6. Grabbe, et al., *Arch Dermatol Res* **28**, 78-84 (1994).
- 10 7. Matsui, et al., *Nature* **347**, 667-9 (1990).
8. Martin, et al., *Cell* **63**, 203-11 (1990).
9. Gattei et al., *Cell Growth & Differentiation.* **7**, 753-63 (1996).
10. Van 'T Hof et al., *FASEB J* **11**, 287-93 (1997).
- 15 11. Ebi, et al., *Blood* **80**, 1454-62 (1992).
12. Suda, et al., *J. Bone Mineral Res.* **12**, 869-79 (1997).
13. Manolagas, et al., *New Engl J Med* **332**, 305-11 (1995).
14. Grano et al., *Proc. Nat. Acad. Sci. USA* **93**, 7644-8 (1996).
15. Grigoriadis, et al., *Science* **266**, 443-8 (1994).
- 20 16. Hayase, et al., *Exp. Hematol.* **25**, 19-25 (1997).
17. Martin, et al., *J. Cellular Biochem.* **56**, 357-66 (1994).
18. Li, et al., *Molecular Endocrinol.* **9**, 805-13 (1995).
19. Alvarez, et al., *Endocrinol.* **128**, 2324-35 (1991).
20. Vilamitjana-Amedee, et al., *In Vitro Cell Dev. Biol.* **29 A**,
25 699-707 (1993).
21. Williams, et al., *J. Biol. Chem.* **271**, 12488-95 (1996).

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications

are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. An inhibitor of osteoblastic stem cell factor binding and/or activity.

5

2. The inhibitor of claim 1, wherein said inhibitor is an antibody.

3. The inhibitor of claim 1, wherein said inhibitor is directed against the membrane associated form of osteoblastic stem cell factor.

10

4. The inhibitor of claim 2, wherein said antibody is directed against the C-terminal end of the stem cell factor protein.

15

5. The inhibitor of claim 4, wherein said C-terminal end comprises the decapeptide, EEDNEISMLQ (SEQ ID No.:1).

20

6. The inhibitor of claim 2, wherein said antibody is non-human.

7. The inhibitor of claim 1, wherein said inhibitor is an antisense oligonucleotide directed against expression of the stem-cell factor.

25

8. The inhibitor of claim 7, wherein said antisense oligonucleotide is directed against the stem-cell factor transcription start site.

5

9. A method of regulating the activity of osteoclasts in an individual in need of such treatment, comprising the step of:
inhibiting the binding and/or activity of osteoblastic stem cell factor in said individual.

10

10. The method of claim 9, wherein said inhibitor is an antibody.

11. The method of claim 9, wherein said antibody is
15 directed against the membrane associated form of osteoblastic stem cell factor.

12. The method of claim 10, wherein said antibody
is directed against the C-terminal end of the stem cell factor
20 protein.

13. The method of claim 12, wherein said C-terminal end comprises the decapeptide, EEDNEISMLQ (SEQ ID NO: 1).

25

14. The method of claim 10, wherein said antibody is non-human.

15. The method of claim 10, wherein said inhibitor is an antisense oligonucleotide directed against expression of the stem-cell factor.

5 16. The method of claim 15, wherein said antisense oligonucleotide is directed against the stem-cell factor transcription start site.

10 17. A pharmaceutical composition comprising the antibody of claim 2 and a pharmaceutically acceptable carrier.

15 18. A pharmaceutical composition comprising the antisense oligonucleotide of claim 7 and a pharmaceutically acceptable carrier.

19. A method of treating a pathophysiological state involving bone loss in an animal in need of such treatment, comprising the step of:

20 administering the pharmaceutical composition of claim 17 to said animal.

20. A method of treating a pathophysiological state in involving bone loss in an animal in need of such treatment, comprising the step of:

25 administering the pharmaceutical composition of claim 18 to said animal.

21. The method of claim 19, wherein said pathophysiological state is selected from the group consisting of osteoporosis, bone cancer and malignant hypercalcemia.

5 22. The method of claim 20, wherein said pathophysiological state is selected from the group consisting of osteoporosis, bone cancer and malignant hypercalcemia.

10 23. A method of diagnosing bone disorders, comprising the step of measuring the activity of osteoblastic stem cell factor in a sample from an individual in need of such diagnosis.

15 24. The method of claim 23, wherein said bone disorders are selected from the group consisting of osteoporosis, hyperparathyroidism, metastatic cancer and hypercalcemia.

SEQUENCE LISTING

<110> Blair, Harry C.
Dong, Sai-Sai
Julian, Bruce A.

5 <120> Regulation of Osteoclast Formation by Inhibition
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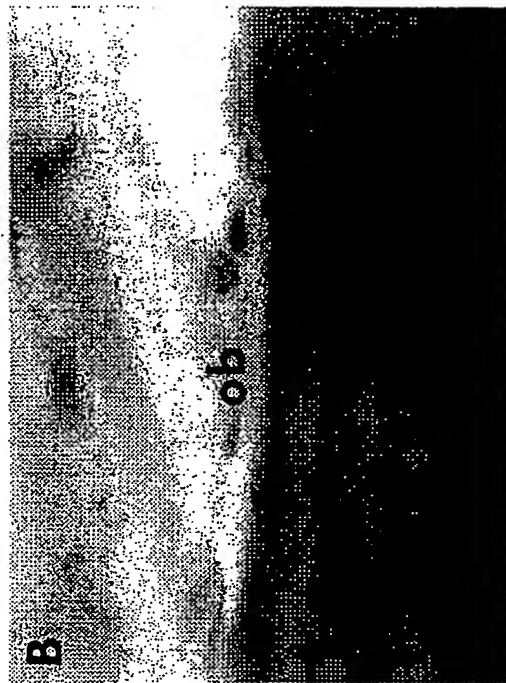


FIGURE 1B

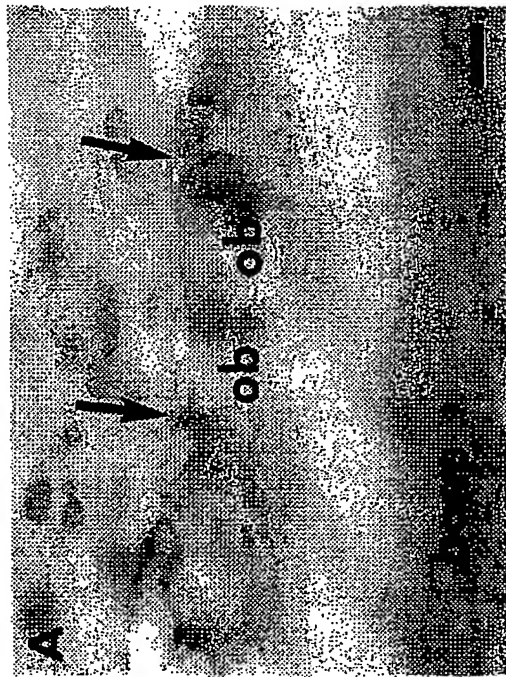


FIGURE 1A

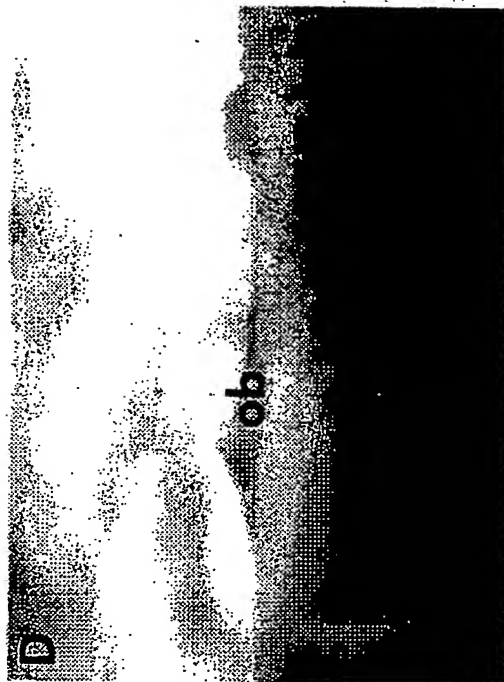


FIGURE 1D



FIGURE 1C

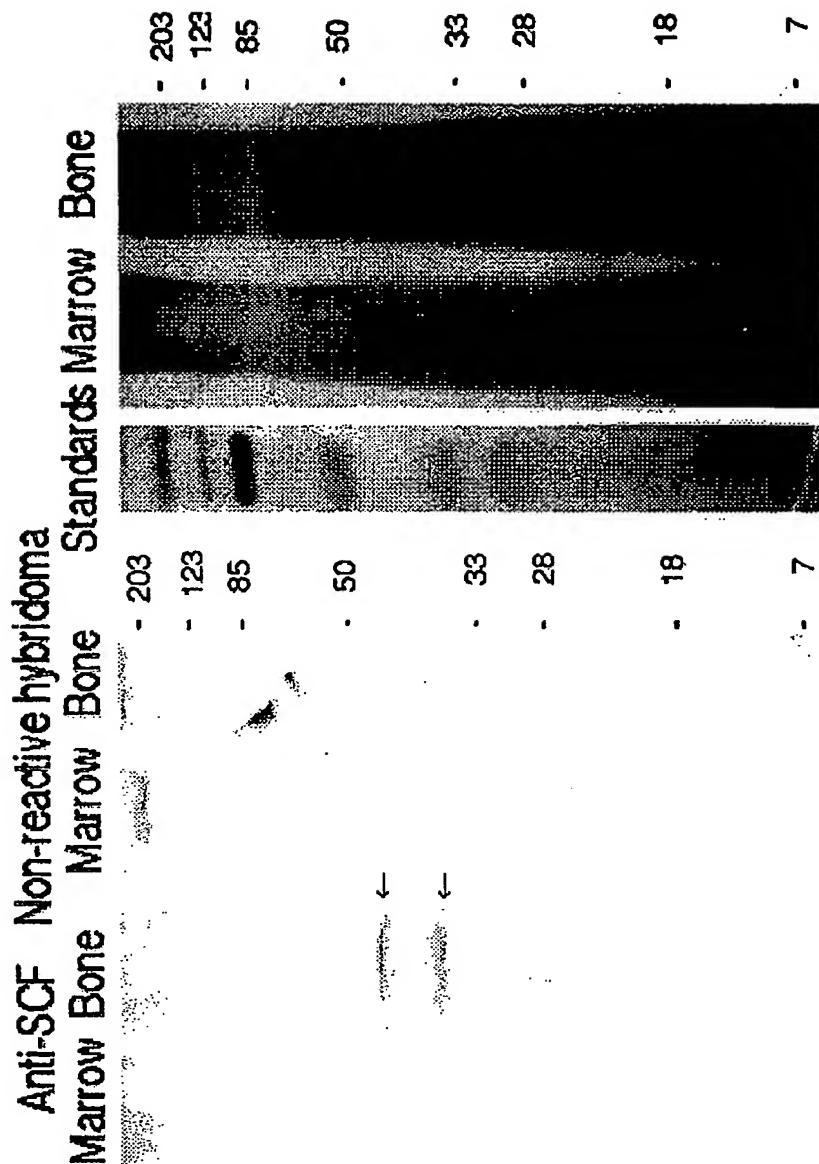
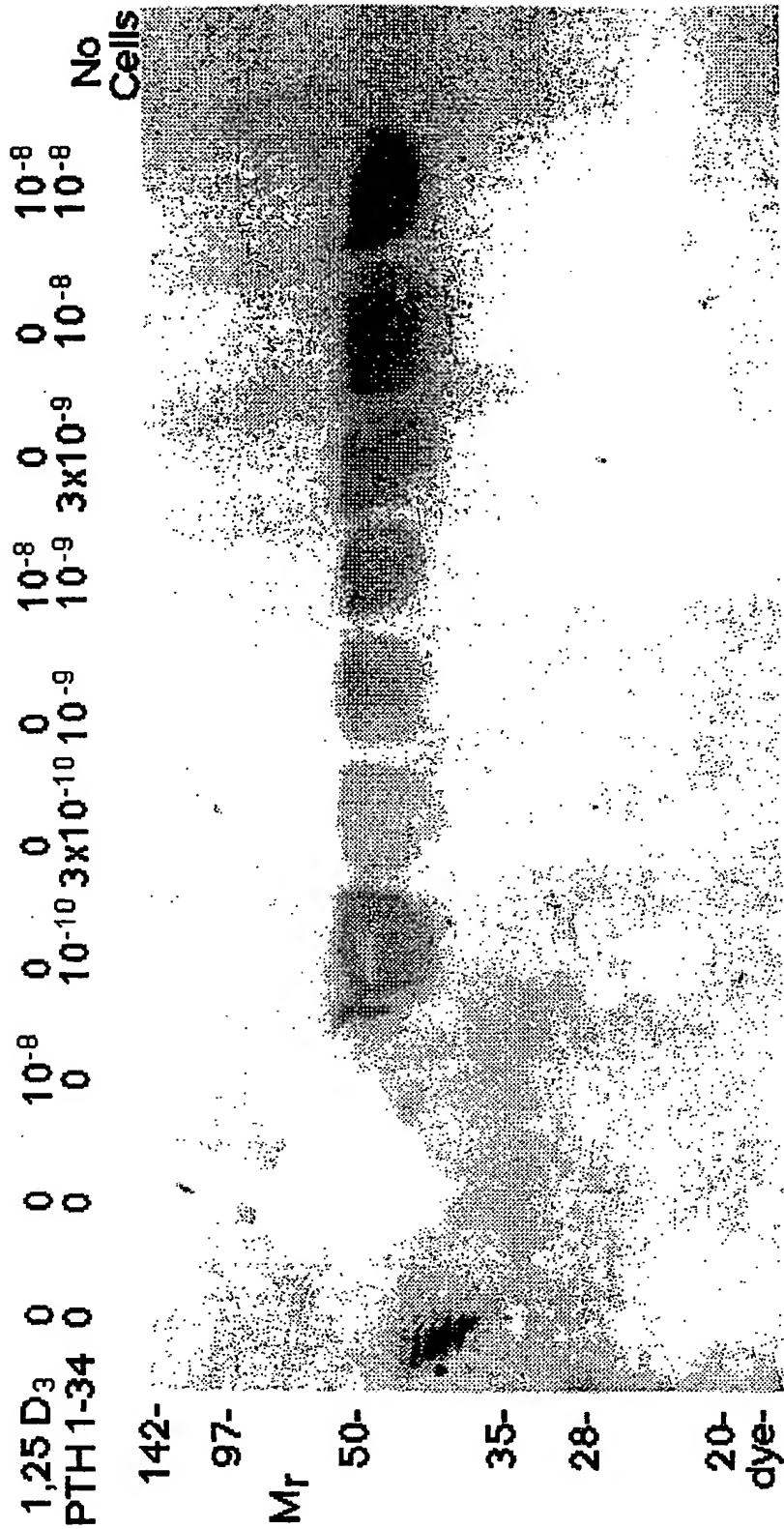


FIGURE 1E



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FIGURE 2A

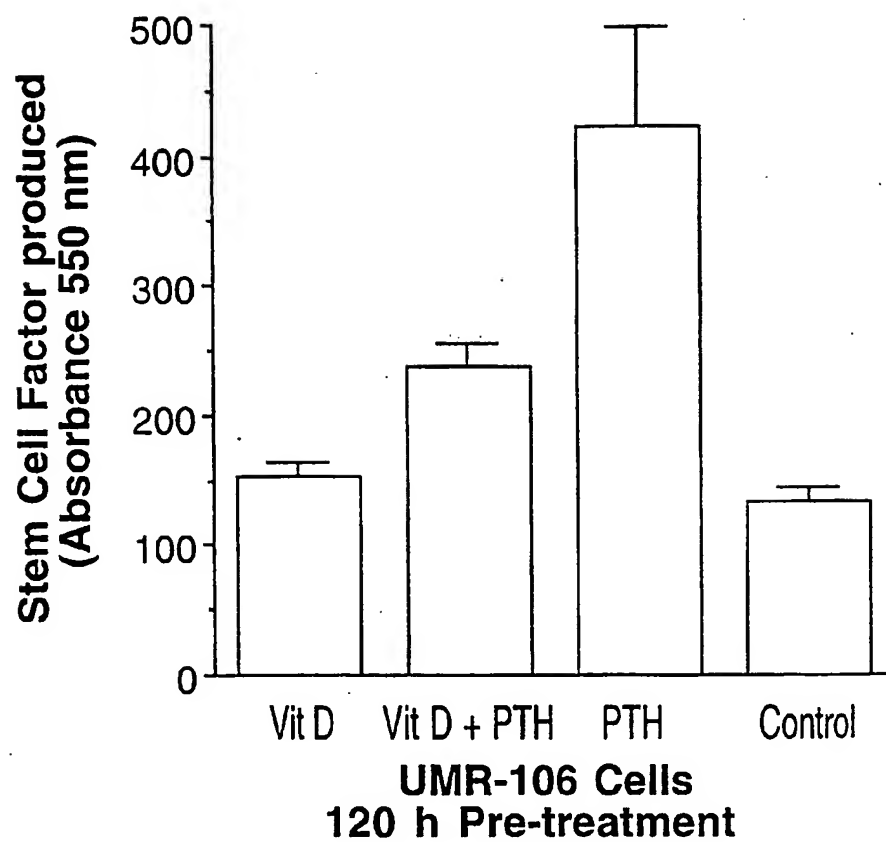


FIGURE 2B

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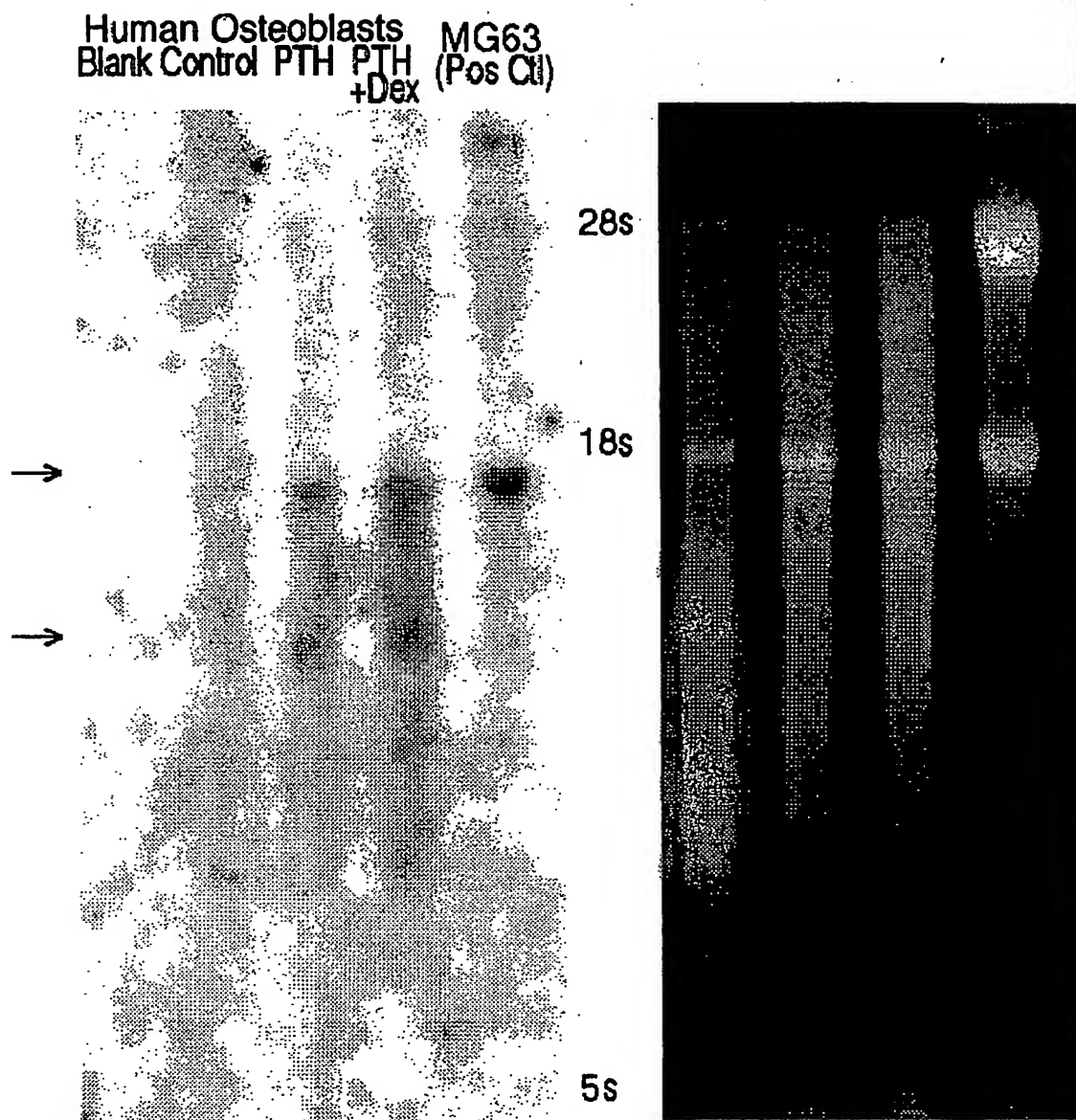


FIGURE 2C

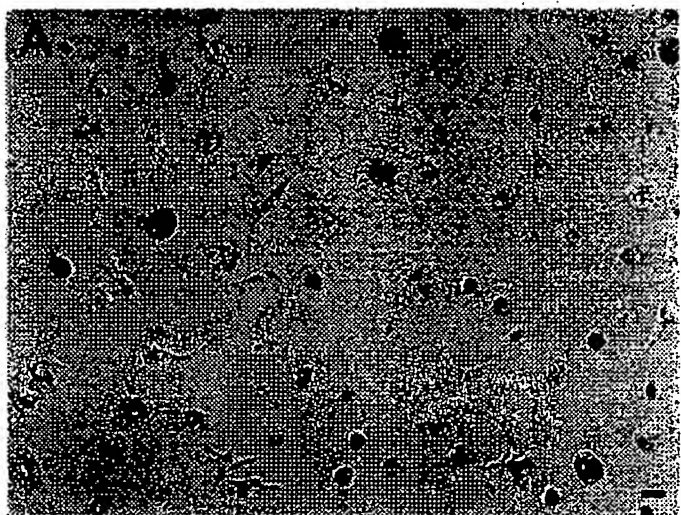


FIGURE 3A

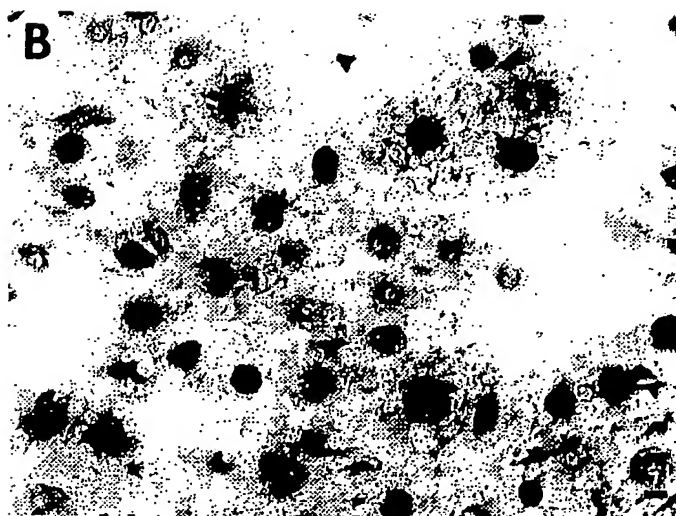


FIGURE 3B

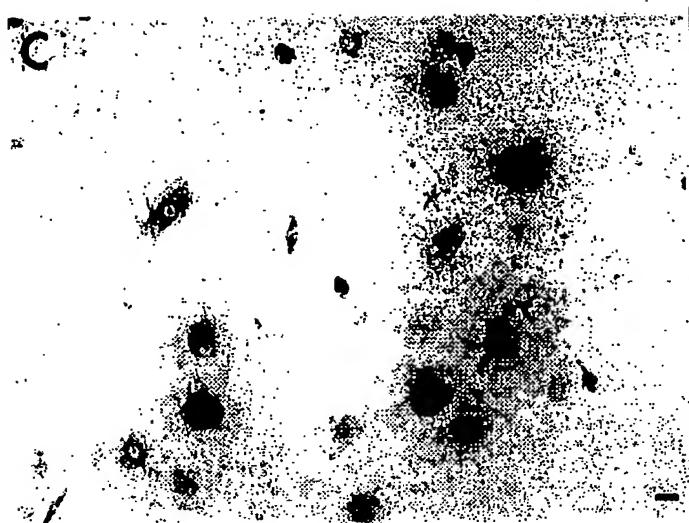


FIGURE 3C

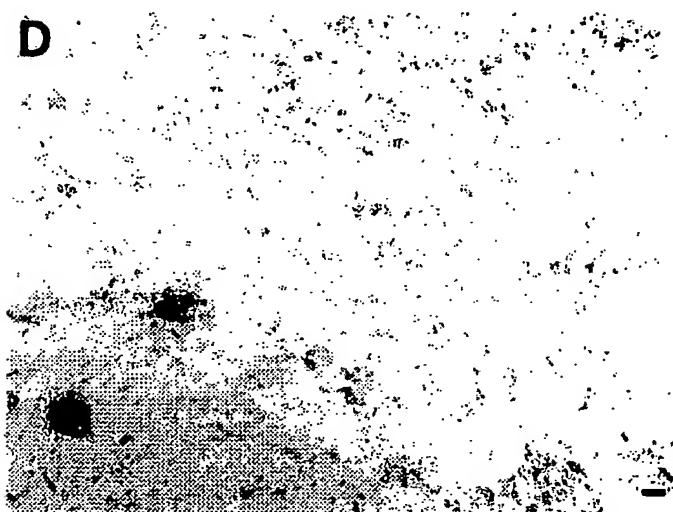


FIGURE 3D

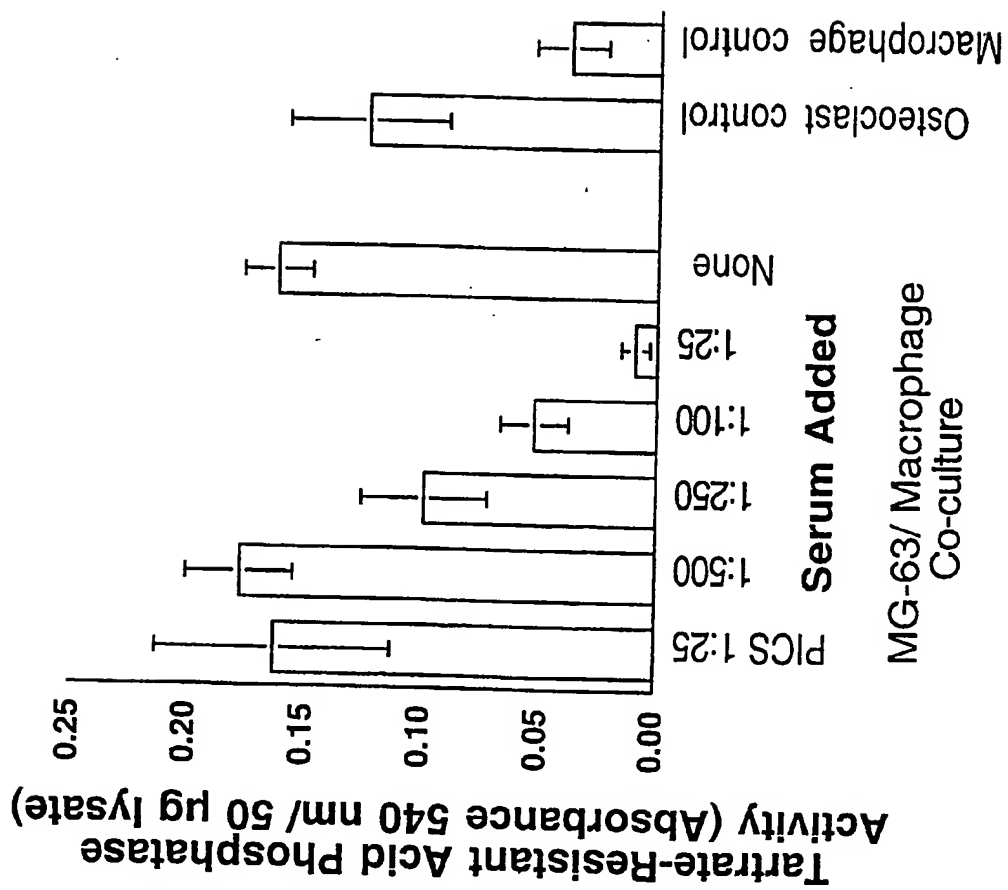


FIGURE 3E-1

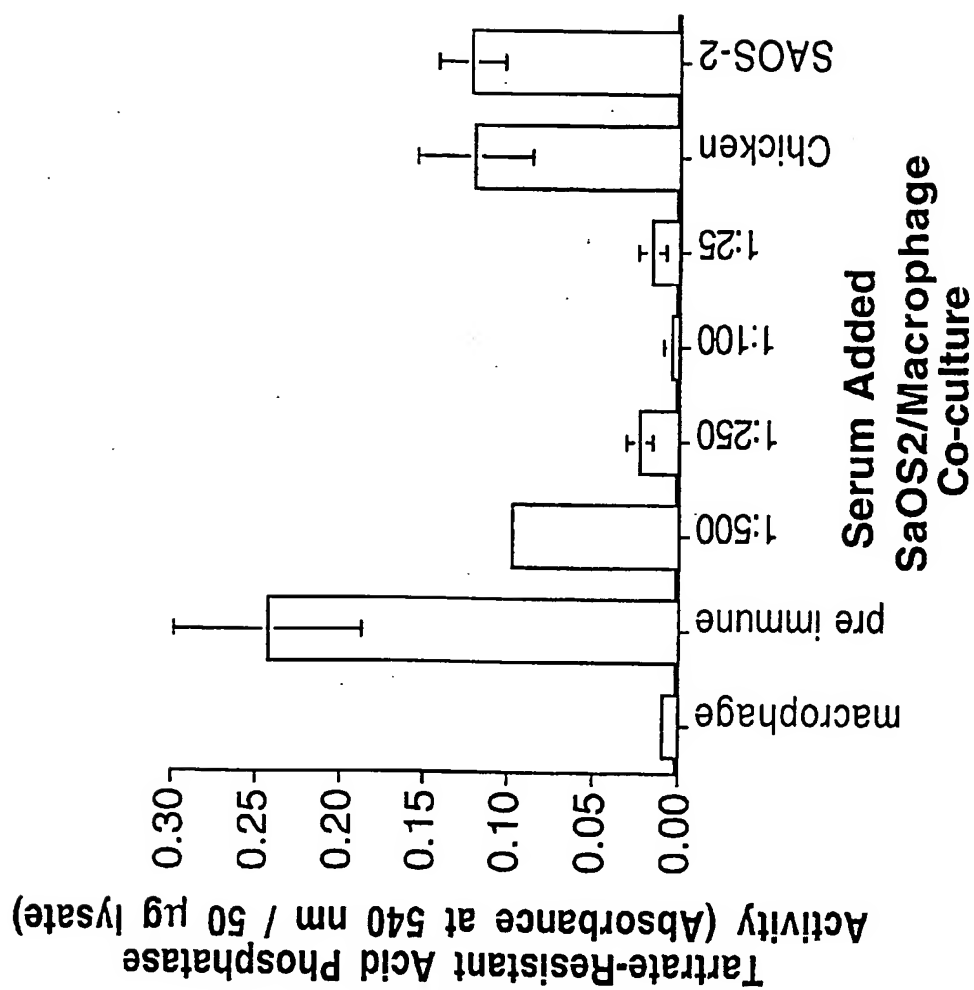


FIGURE 3E-2

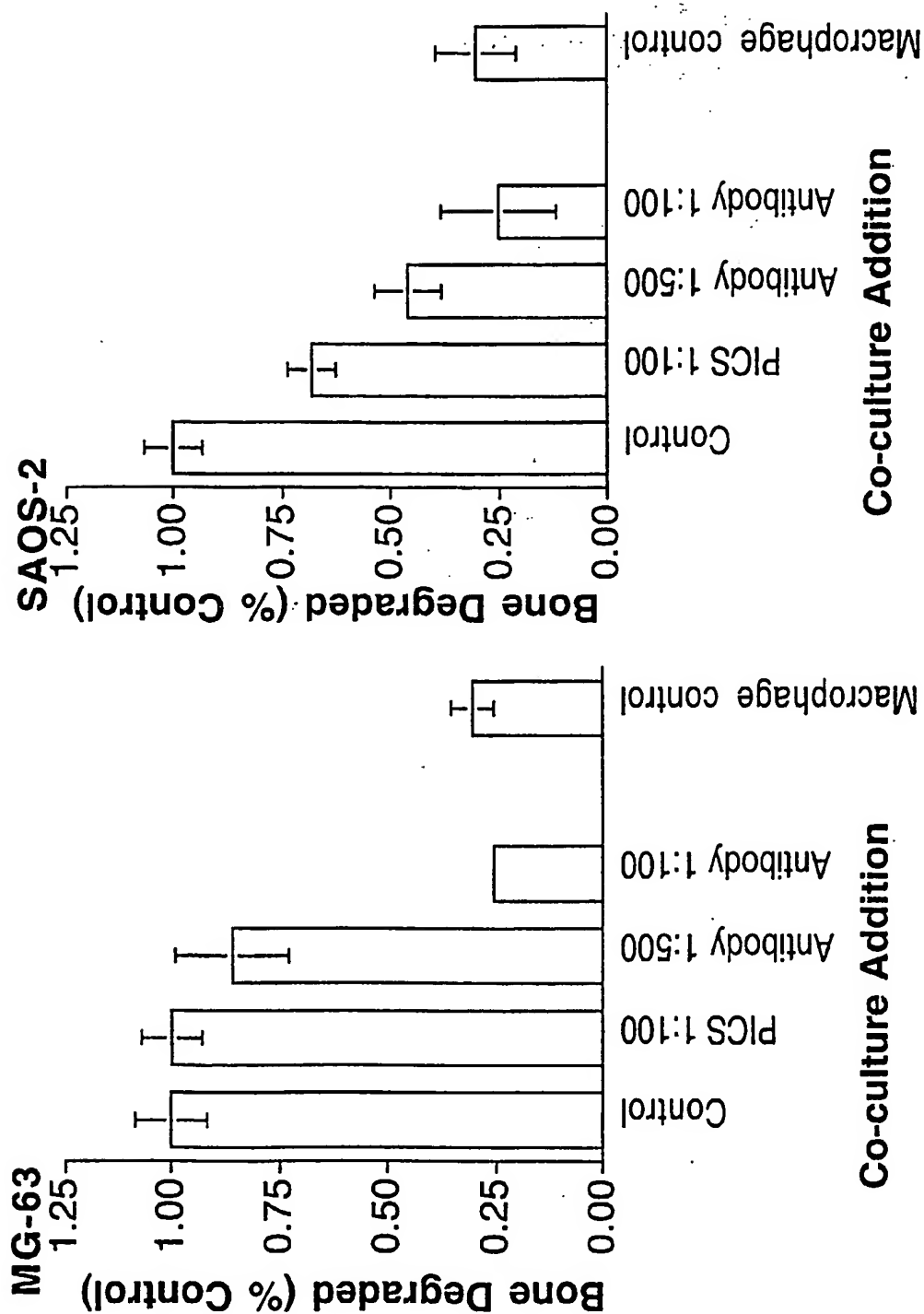


FIGURE 3F

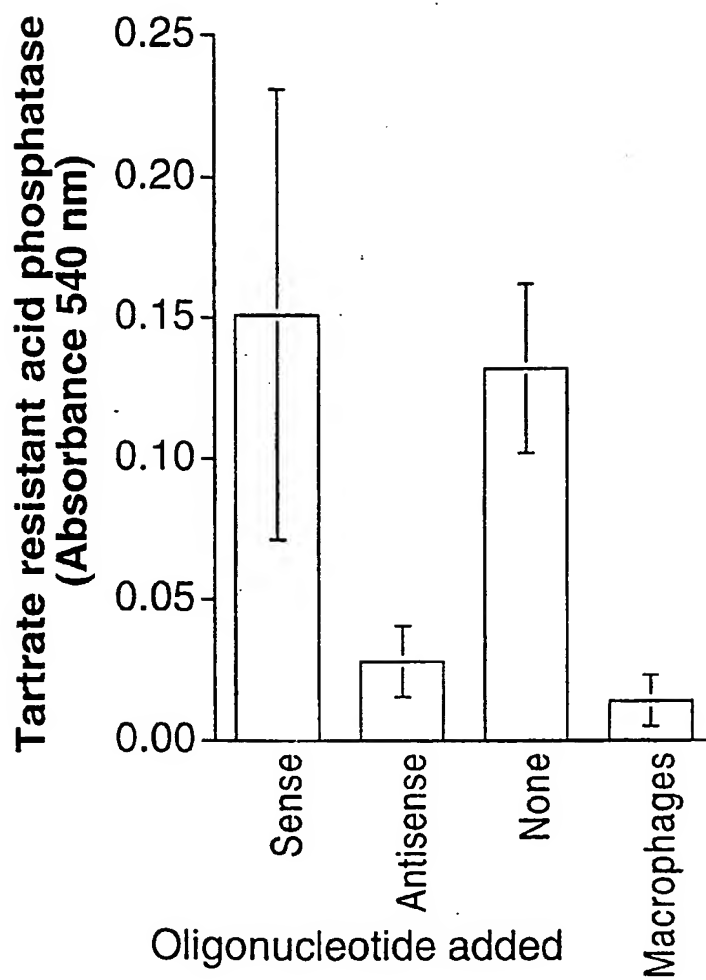


FIGURE 3G

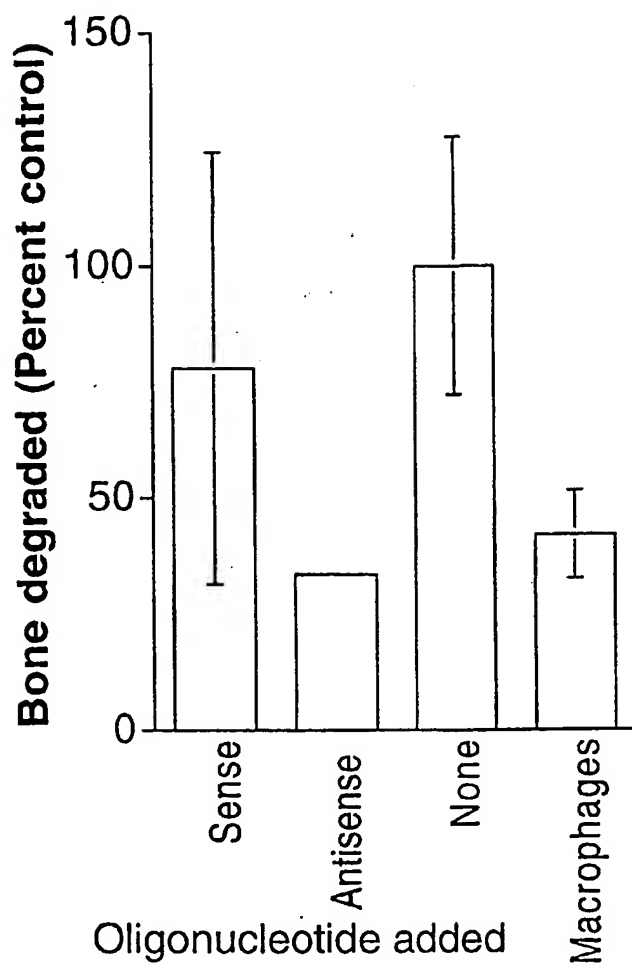


FIGURE 3H

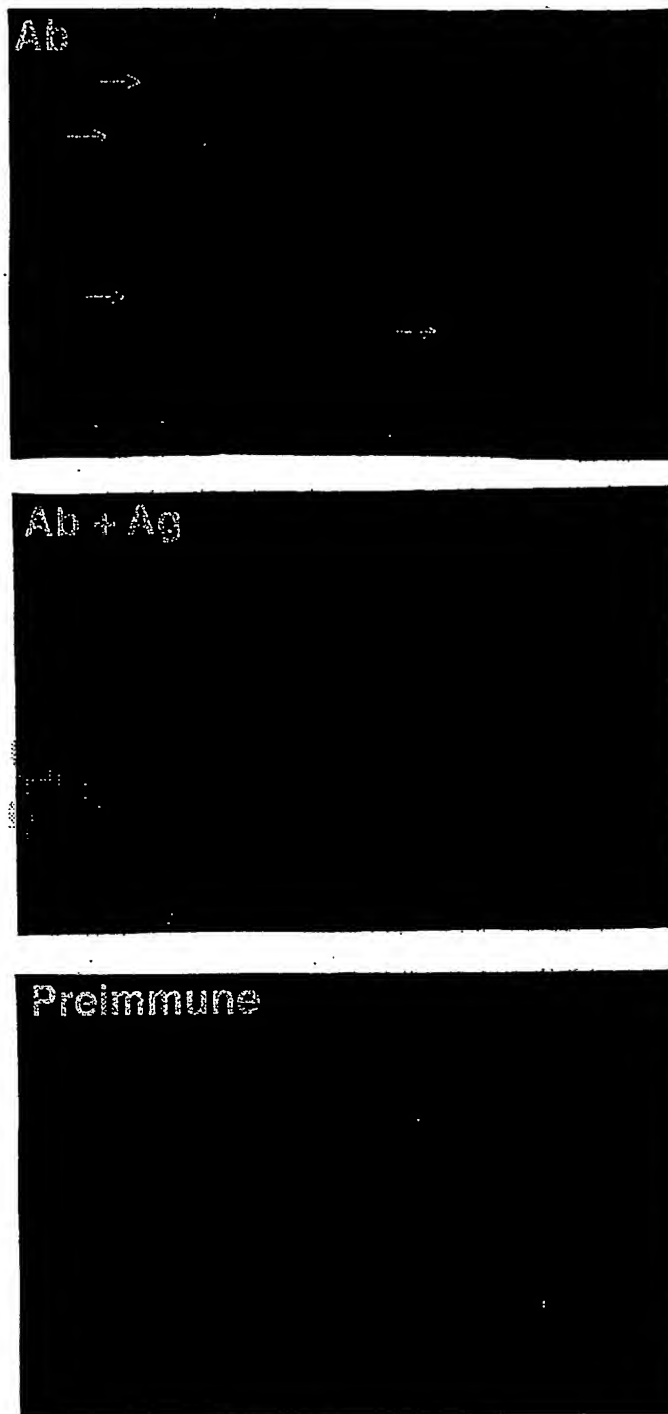


FIGURE 4

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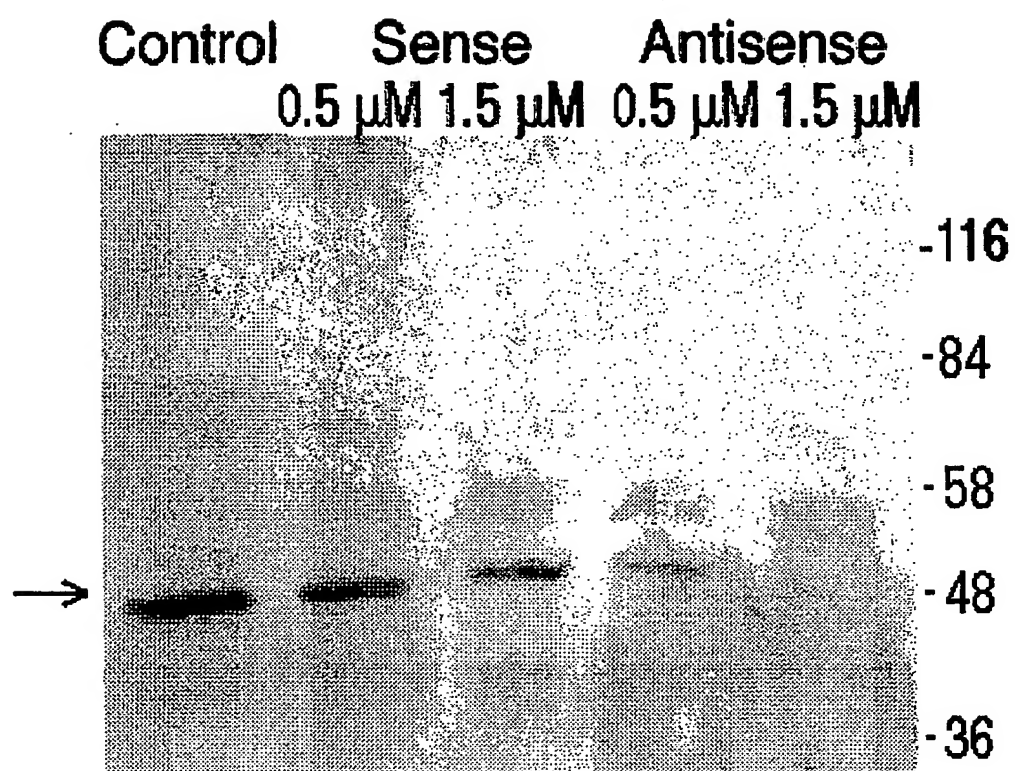


FIGURE 5

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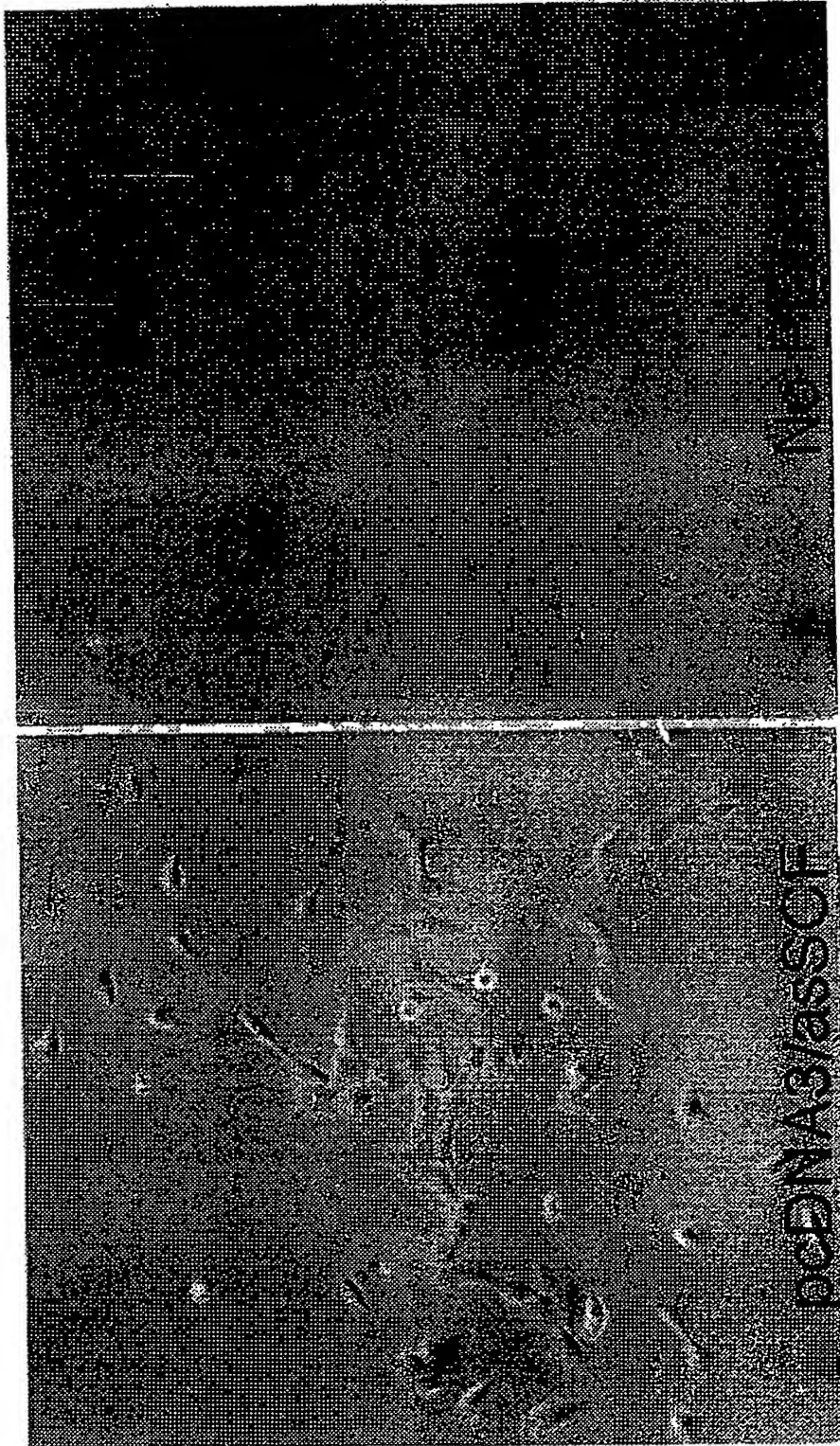


FIGURE 6A

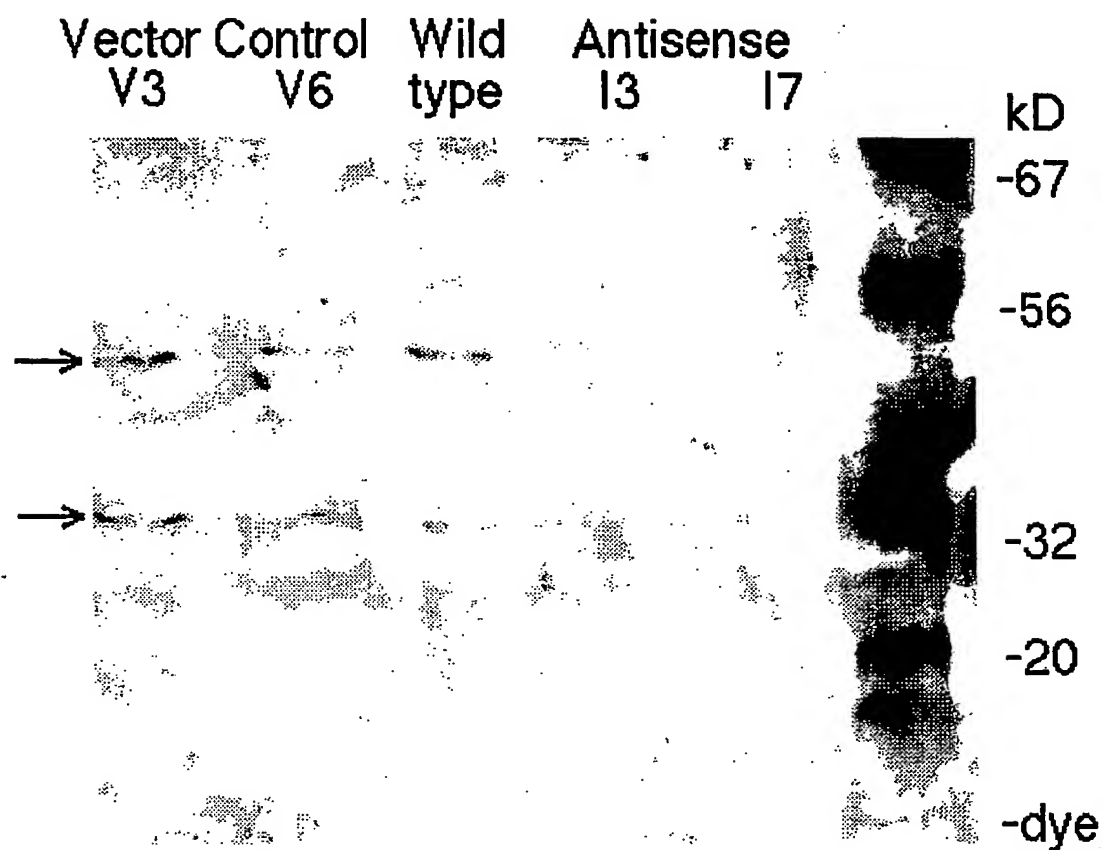


FIGURE 6B

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Bovine	EEDNEISMLQEKEREFQEV	(SEQ ID No. 5)
Feline	EEDNEISMLQEKEREFQEV	(SEQ ID No. 5)
Avian	QEENEISMLQQKEKEHLEV	(SEQ ID No. 6)
Avian (Chicken) soluble	QEENEISMLQQKEKEHLEV	(SEQ ID No. 6)
Avian (Coturnix) membrane	QEENEISMLQQKEKEHLEV	(SEQ ID No. 6)
Canine	EEDNEISMLQEKEREFQEV	(SEQ ID No. 5)
Human	EEDNEISMLQEKEREFQEV	(SEQ ID No. 5)
Human (short form)	EEDNEISMLQEKEREFQEV	(SEQ ID No. 5)
Murine	EEDNEISMLQQKEREFFQEV	(SEQ ID No. 7)
O aries secreted	-----	
Rat partial form	-----	
Porcine	EEDNEISMLQEKEREFQEV	(SEQ ID No. 5)

FIGURE 7

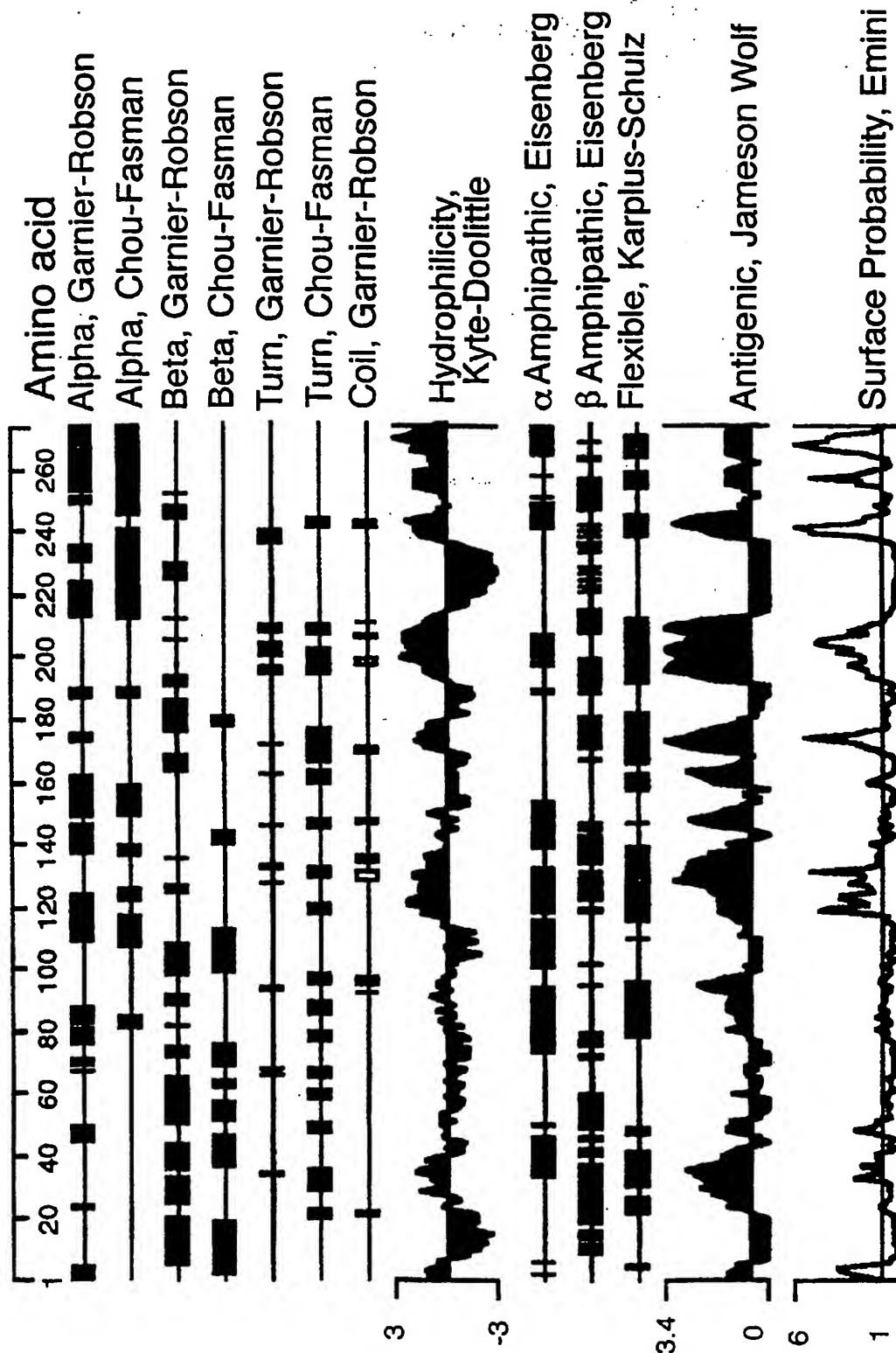


FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18812

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/395, 48/00; C12Q 1/68; C07K 16/00; C07H 21/04
US CL :424/130.1; 435/6, 7.1; 514/44; 530/387.1; 536/23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1; 435/6, 7.1; 514/44; 530/387.1; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, SCISEARCH, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,506,211 A (BARNES et al.) 09 April 1996, see entire document.	1-24
X	HASSAN et al. Stem Cell Factor as a Survival and Growth Factor in Human Normal and Malignant Hematopoiesis. Acta Haematologica. 1996, Vol. 95, pages 257-262, see entire document.	1-8, 17, 18
Y		9-16, 20-24
X	BLAIR et al. Formation Of Human Osteoclasts In Vitro Is Blocked By Antibody To Stem Cell Factor (C-Kit Ligand) Expressed By Osteoblasts Supporting Differentiation. Journal of Bone and Mineral Research. August 1997, Vol. 12 (suppl. 1), page T-380, page 196.	1-6, 17
Y		7-16, 18-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

30 NOVEMBER 1998

Date of mailing of the international search report

24 DEC 1998

Name and mailing address of the ISA/US
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Washington, D.C. 20231

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Telephone No. (703) 308-0196